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**Pro-death NMDA receptor signaling is promoted by the GluN2B C-terminus  
independently of Dapk1**

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38

39 **Abstract**

40 Aberrant NMDA receptor (NMDAR) activity contributes to several neurological disorders, but direct  
41 antagonism is poorly tolerated therapeutically. The GluN2B cytoplasmic C-terminal domain (CTD)  
42 represents an alternative therapeutic target since it potentiates excitotoxic signaling. The key  
43 GluN2B CTD-centred event in excitotoxicity is proposed to involve its phosphorylation at Ser-1303  
44 by Dapk1, that is blocked by a neuroprotective cell-permeable peptide mimetic of the region.  
45 Contrary to this model, we find that excitotoxicity can proceed without increased Ser-1303  
46 phosphorylation, and is unaffected by Dapk1 deficiency *in vitro* or following ischemia *in vivo*.  
47 Pharmacological analysis of the aforementioned neuroprotective peptide revealed that it acts in a  
48 sequence-independent manner as an open-channel NMDAR antagonist at or near the  $Mg^{2+}$  site,  
49 due to its high net positive charge. Thus, GluN2B-driven excitotoxic signaling can proceed  
50 independently of Dapk1 or altered Ser-1303 phosphorylation.

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## 53 Introduction

54 NMDA receptor (NMDAR) -mediated excitotoxicity plays a key role in acute neurological disorders  
55 such as stroke and traumatic brain injury, neuronal loss in Huntington's disease, and is also  
56 implicated in synapto-toxicity in Alzheimer's disease <sup>1-7</sup>. Most NMDARs are comprised of two  
57 obligate GluN1 subunits and two GluN2 subunits <sup>8</sup>, with GluN2A and GluN2B predominant in the  
58 forebrain <sup>9-13</sup>. GluN2 subunits have long, evolutionarily divergent cytoplasmic C-terminal domains  
59 (CTDs) which we have shown can differentially associate with signalling molecules <sup>14-17</sup> and  
60 differentially signal to cell death: the CTD of GluN2B (CTD<sup>2B</sup>) potentiates excitotoxicity more  
61 strongly than that of GluN2A <sup>14</sup>.

62 While multiple pathways contribute to excitotoxicity <sup>18</sup>, the mechanism by which CTD<sup>2B</sup> is  
63 thought to potentiate excitotoxicity is upstream of all of them <sup>6,19,20</sup>. The mechanism is centred on  
64 Ser-1303 of CTD<sup>2B</sup>, within a region of the CTD unique to GluN2B, and with which CaMKII $\alpha$  is known  
65 to interact and phosphorylate <sup>21,22</sup>. It was reported that in response to ischemia or excitotoxic insults,  
66 a different kinase, Dapk1, causes Ser-1303 phosphorylation which increases NMDAR-dependent  
67 ionic flux <sup>19</sup>. Consistent with this, *Dapk1*<sup>-/-</sup> neurons were reported to be resistant to excitotoxicity, and  
68 a cell-permeable peptide mimetic of the CTD<sup>2B</sup> region around Ser-1303 disrupted Ser-1303  
69 phosphorylation and was neuroprotective <sup>19</sup>.

70 Given that the GluN2B-Dapk1 pathway is prominent in contemporary models of excitotoxicity  
71 <sup>6,20</sup> we sought to investigate this pathway further. Dapk1 has not hitherto emerged from proteomic  
72 post-synaptic density screens <sup>23-30</sup>, and we failed to detect it in a recent proteomic analysis of native  
73 NMDAR supercomplexes <sup>17</sup>. Moreover, the use of cell-permeable peptides to draw wide-ranging  
74 mechanistic conclusions can be problematic without extensive controls. We investigated whether  
75 Dapk1-mediated Ser-1303 phosphorylation indeed represents the major reason why CTD<sup>2B</sup>  
76 promotes excitotoxicity signaling better than CTD<sup>2A</sup>, using approaches that include analysis of a  
77 new Dapk1 knockout mouse and the generation of a knock-in mouse with a targeted phospho-  
78 mimetic mutation of the CAMKII $\alpha$ /putative Dapk1 interaction site.

79

## 80 Results

### 81 Excitotoxic insults do not induce GluN2B Ser-1303 phosphorylation

82 We first examined the influence of excitotoxic conditions on GluN2B Ser-1303 phosphorylation in  
83 cortical neurons using a phospho-(Ser-1303)-specific antibody (Millipore 07-398), previously used  
84 and validated by several groups <sup>31-33</sup>.

85 We confirmed that the antibody is capable of detecting changes in Ser-1303 phosphorylation  
86 in neurons: phospho-GluN2B(Ser-1303) levels in cortical neurons, as assayed by western blot using  
87 this antibody, are lowered after incubation of cortical neurons with the general kinase inhibitor  
88 staurosporine, and increased modestly by a cocktail of phosphatase inhibitors okadaic acid and FK-  
89 506 (Figure 1—figure supplement 1a,b, Figure 1—source data 4). As further evidence of specificity,  
90 we found that the antibody completely failed to react with GluN2B in which we had engineered

91 mutations (L1298A/R1300N/S1303D) into the site for a separate study (Figure 1–figure supplement  
92 1c,d).

93 We found that bath application of NMDA at excitotoxic concentrations failed to induce  
94 significant Ser-1303 phosphorylation (Figure 1a,1b, Figure 1–source data 1). At the late timepoint  
95 (60 min, 50  $\mu$ M NMDA) we observed a decline in Ser-1303 phosphorylation (Figure 1a,b, Figure 1–  
96 figure supplement 1e,f, Figure 1–source data 5) as well as a decline in total levels of GluN2B,  
97 consistent with observations of others who have reported partial calpain-mediated cleavage and  
98 degradation of the NMDAR CTD<sup>34,35</sup>. These observations in DIV10 cortical neurons were also  
99 repeated at DIV16 (Figure 1–figure supplement 1g,h, Figure 1–source data 6). We also saw no  
100 increase in Ser-1303 phosphorylation in response to oxygen-glucose deprivation (OGD) (Figure  
101 1c,d, Figure 1–source data 2), contrary to previous reports<sup>19</sup>.

102 To determine whether Dapk1 plays any role in the GluN2B Ser-1303 phosphorylation status,  
103 we obtained a *Dapk1*<sup>-/-</sup> mouse line, created by the International Mouse Phenotyping Consortium by  
104 targeted deletion of exon 4 on a C57Bl/6 background (the same strain as the *Dapk1*<sup>-/-</sup> mouse  
105 generated by Tu et al (2010)). The mice had normal fertility, viability and body weight  
106 (<http://www.mousephenotype.org>, MGI:1916885). We confirmed that *Dapk1*<sup>-/-</sup> neurons expressed no  
107 Dapk1 (Figure 1e). We compared GluN2B phospho-Ser-1303 levels in cortical neurons obtained  
108 from *Dapk1*<sup>-/-</sup> and *Dapk1*<sup>+/+</sup> littermates and found no difference in basal levels, nor any difference in  
109 the lowered level that we observe at longer periods of NMDA exposure (Figure 1e,f, Figure 1–  
110 source data 3). Thus in our hands, Dapk1 does not influence GluN2B Ser-1303 phosphorylation  
111 status under basal or excitotoxic conditions.

### 113 **Excitotoxic and ischemic neuronal death can proceed independently of Dapk1**

114 We next addressed the more general point of the role of Dapk1 in excitotoxic neuronal death.  
115 Compared to cortical neurons cultured from their wild-type littermates, we observed no difference in  
116 NMDAR-dependent excitotoxic neuronal death in *Dapk1*<sup>-/-</sup> neurons at either DIV10 or DIV16 (Figure  
117 2a, Figure 2–source data 1; Figure 2b, Figure 2–source data 2) and no difference in OGD-induced  
118 neuronal death (Figure 2c, Figure 2–source data 3), contrary to previous reports. NMDAR currents  
119 were also no different in *Dapk1*<sup>-/-</sup> vs. *Dapk1*<sup>+/+</sup> neurons (Figure 2d, Figure 2–source data 4).

120 We then studied the influence of Dapk1 deficiency on ischemic neuronal death *in vivo*. We  
121 employed a model of transient global ischemia model (bilateral common carotid artery occlusion)  
122 used previously to show a protective effect of Dapk1 deficiency<sup>19</sup>. Adult mice exposed to a transient  
123 (20 min) period of global ischemia showed characteristic selective neuronal death within the  
124 hippocampus, particularly the CA1 and CA2 regions. However, *Dapk1*<sup>-/-</sup> and *Dapk1*<sup>+/+</sup> mice exhibited  
125 similar levels of infarction (Figure 2e-h, Figure 2–source data 5), contrary to previous reports<sup>19</sup>.  
126 These observations collectively indicate that excitotoxic and ischemic neuronal death *in vitro* and *in*  
127 *vivo* can proceed normally in the absence of Dapk1.

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### **TAT-NR2B<sub>CT</sub> is a direct NMDAR antagonist**

In support of the Dapk1 hypothesis for CTD<sup>2B</sup>-derived excitotoxicity, a cell-permeable (TAT-fused) peptide mimetic of the GluN2B amino acids 1292-1304 (TAT-KKNRNKLRRQHSY: TAT-NR2B<sub>CT</sub>) was reported to prevent NMDAR-dependent GluN2B Ser-1303 phosphorylation, and excitotoxicity<sup>19</sup>. We observed that 50  $\mu$ M TAT-NR2B<sub>CT</sub>, the concentration used previously<sup>19</sup>, was toxic to neurons (Figure 3—figure supplement 1a, Figure 3—source data 4), so we used a concentration 10 times lower (5  $\mu$ M). We found that 5  $\mu$ M TAT-NR2B<sub>CT</sub> completely prevents NMDA-induced excitotoxicity (Figure 3—figure supplement 1b, Figure 3—source data 5). This was surprising, given that we did not see a role for Dapk1 in excitotoxicity (Figure 2). However, further analysis revealed the explanation: at 5  $\mu$ M, TAT-NR2B<sub>CT</sub> potently inhibited NMDAR currents, acting immediately and without any need for a preincubation period (Figure 3a-d, Figure 3—source data 1), and in a manner that was not readily washed out upon removal of peptide (data not shown). TAT-NR2B<sub>CT</sub> was custom synthesized for our studies by Genscript, and we found that NR2B<sub>CT</sub>(1292-1304)-TAT, a pre-made peptide sold by Merck Millipore was a similarly potent NMDAR antagonist (n=8, Figure 3—figure supplement 1c). A scrambled version of TAT-NR2B<sub>CT</sub> (TAT-sNR2B<sub>CT</sub>) was similarly neuroprotective and similarly antagonistic at the NMDAR (Figure S3b, Figure 3a-d). One potential explanation for the NMDAR antagonistic properties of TAT-NR2B<sub>CT</sub> is the high positive charge of the peptide (+15 at neutral pH). To investigate this, we designed an arginine-rich peptide of high net positive charge (+15-same as TAT-NR2B<sub>CT</sub>) of sequence: RRR TQN RRN RRT SRQ NRR RSR RRR) which strongly antagonized NMDAR currents, and another peptide of net neutral charge (NIN IHD VKV LPG GMI KSN DGP PIL), which had a much weaker effect (Figure 3a, Figure 3—source data 1). Taken together these data suggest that the net positive charge of TAT-NR2B<sub>CT</sub> is primarily responsible for its NMDAR-antagonistic properties. We hypothesized that TAT-NR2B<sub>CT</sub> may be an open channel blocker drawn partly into the pore by its high net positive charge and bind near the internal Mg<sup>2+</sup> binding site. Consistent with this, the presence of Mg<sup>2+</sup> (a pore blocker) reduced the effectiveness of TAT-NR2B<sub>CT</sub>'s antagonism (Figure 3e, Figure 3—source data 2). Another prediction of this hypothesis is that TAT-NR2B<sub>CT</sub> would be more effective at antagonising NMDARs under open-channel conditions. To test this, NMDAR currents were measured, after which neurons were incubated in TAT-NR2B<sub>CT</sub> (5  $\mu$ M) for 60 s in the presence of zero glycine+100  $\mu$ M AP5 ("block (1)") to ensure minimal channel opening (closed channel conditions). 60 s was chosen because under conditions of NMDAR agonism this is sufficient time to achieve maximal blockade. After 60 s, both TAT-NR2B<sub>CT</sub> and AP5 were removed from the bathing medium and NMDAR currents were subsequently re-measured (150  $\mu$ M NMDA + 100  $\mu$ M glycine, zero Mg<sup>2+</sup>). The very slow off-rate of the TAT-NR2B<sub>CT</sub> enabled the peptide's effects on currents to be measured in the absence of the peptide in the medium. TAT-NR2B<sub>CT</sub> was then applied for a second 60 s ("block (2)") either under the same "closed channel conditions" or under "open channel conditions" (100  $\mu$ M glycine, 150  $\mu$ M

167 NMDA). After 60 s TAT-NR2B<sub>CT</sub> was removed from the bathing medium and NMDAR currents  
168 measured for a 3rd time. The NMDAR current remaining at the 2nd and 3rd measurements was  
169 calculated as a fraction of the initial current. We found that the 2<sup>nd</sup> peptide incubation (block (2))  
170 significantly increased the proportion of NMDAR inhibition when it occurred under open-channel  
171 conditions, but not under closed channel conditions (Figure 3f, Figure 3—source data 3, further  
172 evidence in favour of a pore-centred binding site for TAT-NR2B<sub>CT</sub>. Thus, the unintended NMDAR  
173 antagonistic properties TAT-NR2B<sub>CT</sub> explain its anti-excitotoxic effects.

174

## 175 Discussion

176 Dapk1-mediated GluN2B Ser-1303 phosphorylation, and consequent enhancement of toxic Ca<sup>2+</sup>  
177 influx through extrasynaptic NMDARs lies at the heart of current models of excitotoxicity and of the  
178 central role of the GluN2B CTD in this process <sup>6,20</sup>, but our study suggests that this needs to be re-  
179 appraised. Our observations regarding the (lack of) impact of Dapk1 gene deletion on neuronal  
180 vulnerability to excitotoxic and ischemic conditions is at odds with previous reports <sup>19</sup>. The *Dapk1*<sup>-/-</sup>  
181 mouse that we used was generated independently of the one generated by Tu et al., although there  
182 is no *a priori* reason why the two lines should behave differently at this fundamental level,  
183 particularly given the very similar genetic background (C57BL/6).

184 The potent inhibition of NMDAR currents by TAT-NR2B<sub>CT</sub> at a concentration up to 100 times  
185 lower than that used previously <sup>19</sup> suggests a simple explanation for its neuroprotective effects  
186 independent of Dapk1. We are unable to explain why we observed similar effects of TAT-NR2B<sub>CT</sub>  
187 and its scrambled version, while a selective effect of TAT-NR2B<sub>CT</sub> was previously reported <sup>19</sup>. Both  
188 scrambled versions employed had identical sequences, and the potent NMDAR antagonistic  
189 properties of our scrambled peptide are consistent with its neuroprotective properties.

190 One outstanding question is the basis for the modestly reduced excitotoxicity in young  
191 neurons when NMDARs lack the GluN2B CaMKII site. We know that NMDAR currents are  
192 unaffected, as is the proportion of NMDARs at synaptic vs. extrasynaptic sites, an important factor  
193 in excitotoxicity <sup>36</sup>, are unaltered. This is consistent with other studies which have concluded that  
194 mutation of this site does not affect NMDAR biophysical properties <sup>37,38</sup>. More generally, the basis  
195 for CTD<sup>2B</sup>-mediated excitotoxicity <sup>14</sup> remains incompletely understood. Exchanging the CTD of  
196 GluN2B with that of GluN2A by targeted exon exchange reduces vulnerability to excitotoxicity <sup>14</sup>,  
197 and performing the reciprocal swap increases vulnerability (SM and GEH, unpublished  
198 observations), strongly supportive of a key role for CTD<sup>2B</sup>. An ongoing avenue of investigation is  
199 focussed on understanding the extent to which the composition of the native NMDAR signaling  
200 complex is altered by manipulating the endogenous GluN2 CTDs in our panel of knock-in mice. We  
201 hypothesize that alterations to the complex may disturb signaling to pro-death events such as NO  
202 production, NADPH oxidase activation, oxidative stress, calpain activation and mitochondrial Ca<sup>2+</sup>  
203 overload <sup>6,20,36,39-43</sup>. Of note, we recently showed that the CTD of GluN2B (as opposed to that of  
204 GluN2A) is critically required for formation of 1.5 MDa NMDAR supercomplexes <sup>17</sup>. Thus, regions

205 unique to GluN2B (of which the CaMKII site is one) play a role in higher order signal complex  
206 assembly and this may underlie the key role of CTD<sup>2B</sup> in downstream excitotoxicity<sup>14</sup>.

207

## 208 **Materials and Methods**

209

### 210 ***Neuronal culture, Dapk<sup>-/-</sup> mice, induction of excitotoxicity and oxygen-glucose deprivation***

211 Cortical mouse neurons were cultured as described<sup>44</sup> at a density of between 9-13 x 10<sup>4</sup> neurons  
212 per cm<sup>2</sup> from E17.5 mice with Neurobasal growth medium supplemented with B27 (Invitrogen,  
213 Paisley, UK). Stimulations of cultured neurons were done in most cases after a culturing period of 9-  
214 11 days during which neurons develop a network of processes, express functional NMDA-type and  
215 AMPA/kainate-type glutamate receptors, and form synaptic contacts. Other experiments were  
216 performed at DIV 16. Dapk<sup>-/-</sup> mice (colony name: H-Dapk1-B11-TM1B, MGI Allele Name:  
217 Dapk1tm1b(EUCOMM)Hmgu, RID:MGI:5756958) were generated by MRC Harwell from targeted  
218 ES cells made by The European Conditional Mouse Mutagenesis Program, as part of the  
219 International Mouse Phenotyping Program. Dapk<sup>-/-</sup> genotyping reactions were performed using the  
220 following primers: A = 5-AGAGAACTGAGGCACCTGG -3', B =, 5'-  
221 CATCCAAAGTCCACAGCCAC-3', C=5'-CCAGTTGGTCTGGTGTCA-3' Primer pair A-B recognised  
222 the wild-type allele and amplified a product of 322 bp. Primer pair B-C recognised the mutant allele  
223 corresponding to a product of 468 bp. PCR reactions were performed using the following cycling  
224 conditions: 15 min at 95°C; 36 cycles of 45 s at 94°C, 45 s at 60°C and 1 min at 72°C; and 10 min at  
225 72°C.

226 To apply an excitotoxic insult, neurons were first placed overnight into a minimal defined  
227 medium<sup>45</sup> containing 10% MEM (Invitrogen), 90% Salt-Glucose-Glycine (SGG) medium (<sup>46</sup>; SGG:  
228 114 mM NaCl, 0.219 % NaHCO<sub>3</sub>, 5.292 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM HEPES, 1 mM  
229 Glycine, 30 mM Glucose, 0.5 mM sodium pyruvate, 0.1 % Phenol Red; osmolarity 325 mosm/l,<sup>47</sup>).  
230 Where used, TAT-NR2B<sub>CT</sub> or TAT-sNR2B<sub>CT</sub> (5 µM) was incubated for 1h prior to the excitotoxic  
231 insult. Neurons were then treated with NMDA (Tocris Bioscience, Bristol, UK) at the indicated  
232 concentrations for 1 h, after which medium was changed to NMDA-free. After a further 23 h,  
233 neurons were fixed and subjected to DAPI staining and cell death quantified by counting (blind) the  
234 number of shrunken, pyknotic nuclei as a percentage of the total. To induce oxygen-glucose  
235 deprivation, a previously described approach was used<sup>48,49</sup>. Briefly, cells were washed and  
236 incubated in glucose-free SGG (see formulation above, but with glucose replaced by mannitol) that  
237 had been previously degassed with 95% N<sub>2</sub>-5% CO<sub>2</sub>. The cells were then placed in an anoxic  
238 modular incubator chamber for 120 min (as compared to cells washed and incubated in normoxic  
239 glucose-containing SGG). For analysis of excitotoxicity, approximately 800-1000 cells were  
240 analysed per condition, per replicate (repeated across several replicates), the observer blind to  
241 genotype and experimental condition.

242



## **Electrophysiological recording and analysis**

Coverslips containing cortical neurons were transferred to a recording chamber perfused (at a flow rate of 3-5 ml/min) with an external recording solution composed of (in mM): 150 NaCl, 2.8 KCl, 10 HEPES, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 glucose and 0.1 glycine, pH 7.3 (320-330 mOsm). Patch-pipettes were made from thick-walled borosilicate glass (Harvard Apparatus, Kent, UK) and filled with a K-gluconate-based internal solution containing (in mM): potassium gluconate 141, NaCl 2.5, HEPES 10, EGTA 11; pH 7.3 with KOH). Electrode tips were fire-polished for a final resistance ranging between 4-8 MΩ. Currents were recorded at room temperature (21 ± 2°C) using an Axopatch 200B amplifier (Molecular Devices, Union City, CA). Neurons were voltage-clamped at -60 mV or +40 mV as indicated, and recordings were rejected if the holding current was greater than -100 pA (-60 mV only) or if the series resistance drifted by more than 20% of its initial value (<25 MΩ). All NMDA currents were evoked by 150 μM NMDA + 100 μM glycine except figure 2D where 50 μM NMDA + 100 μM glycine was used. Whole-cell currents were analyzed using WinEDR v3.2 software (John Dempster, University of Strathclyde, UK). The approximate number of cells to be recorded was estimated in order to detect a 25% difference in the parameter under study, powered at 80%, based on the standard deviation of data previously published by the laboratory<sup>14,50,51</sup>.

To determine the ifenprodil-sensitivity of neurons, whole cell NMDA currents were recorded (as described above) followed by the inclusion of 3 μM ifenprodil in the recording solution for a blocking period of 90 seconds. The whole cell NMDA current was re-assessed, with 3 μM ifenprodil included, and the percentage block calculated. A similar protocol was used to determine the competing effect of Mg<sup>2+</sup> and TAT-NR2B<sub>CT</sub> except a blocking period of 60 seconds was used and TAT-NR2B<sub>CT</sub> was not included when NMDA currents were re-assessed; this may have led to a small washout but we deemed this as negligible due to the slow-off rate of NR2BCT. The membrane potential-dependency of TAT-NR2B<sub>CT</sub>-induced NMDAR antagonism was determined by applying the peptide for 50-60 seconds after initial steady state at both - 60mV or + 40 mV.

To investigate the use dependency of TAT-NR2B<sub>CT</sub>, we minimized the possibility of the NMDAR channel opening by spontaneous release of glutamate by removing glycine from the ACSF and co-applying 100 μM AP5. Glycine was added back to the ACSF to measure NMDA currents and to facilitate the block of TAT-NR2B<sub>CT</sub> in the open channel configuration.

## **Western blotting**

Western blotting was performed as described<sup>52</sup>. In order to minimize the chance of post-translational modifications during the harvesting process, neurons were lysed immediately after stimulation in 1.5x LDS sample buffer (NuPage, Life Technologies) and boiled at 100°C for 10 min. Approximately 10 μg of protein was loaded onto a precast gradient gel (4-16%) and subjected to electrophoresis. Western blotting onto a PVDF membrane was then performed using the Xcell Surelock system (Invitrogen) according to the manufacturer's instructions. Following the protein transfer, the PVDF membranes were blocked for 1 h at room temperature with 5% (w/v) non-fat

281 dried milk in TBS with 0.1% Tween 20. The sample size was calculated based on previous  
282 experimental observations of reporting the effect and standard deviation of NMDA-induced Ser-  
283 1303 phosphorylation<sup>19</sup>. The membranes were incubated at 4°C overnight with the primary  
284 antibodies diluted in blocking solution: Anti phospho-(Ser-1303) GluN2B (1: 2000, Millipore), anti-  
285 Dapk1 (1:8000, Sigma), anti-GluN2B (C-terminus, 1:8000, BD Transduction Laboratories), anti-beta  
286 actin (1:200000, Abcam). For visualisation of Western blots, HRP-based secondary antibodies were  
287 used followed by chemiluminescent detection on Kodak X-Omat film. Western blots were digitally  
288 scanned and densitometric analysis was performed using Image J. All analysis of GluN2B  
289 phosphorylation was normalized to total GluN2B.

290

### 291 ***Bilateral common carotid artery occlusion***

292 Mice were housed in individually-ventilated cages (in groups of up to five mice) under specific  
293 pathogen-free conditions and standard 12 h light/dark cycle with unrestricted access to food and  
294 water. All experiments using live animals were conducted under the authority of UK Home Office  
295 project and personal licences and adhered to regulations specified in the Animals (Scientific  
296 Procedures) Act (1986) and Directive 2010/63/EU and were approved by both The Roslin Institute's  
297 and the University of Edinburgh's Animal Welfare and Ethics Committees. Experimental design,  
298 analysis and reporting followed the ARRIVE guidelines (<https://www.nc3rs.org.uk/arrive-guidelines>)  
299 where possible. The sample size was calculated based on the experimental observations of  
300 reporting the effect and standard deviation of BCCAO-induced neuronal loss in both wild-type and  
301 *Dapk1*<sup>-/-</sup><sup>19</sup>, whose experimental observations using n=7 per genotype we retrospectively calculated  
302 were powered at >99%.

303 Transient bilateral common carotid artery occlusion (BCCAO) was performed in *Dapk1*<sup>-/-</sup>  
304 and wild-type male control mice under isoflurane anaesthesia (with O<sub>2</sub> and N<sub>2</sub>O). The operator was  
305 unaware of genotype. Core body temperature was maintained at 37 ± 0.5°C throughout the  
306 procedure with a feedback controlled heating blanket (Harvard Apparatus, UK). Both common  
307 carotid arteries were exposed and dissected from surrounding tissues and occluded by application  
308 of an aneurysm clip for 20 min. Clips were removed, the neck wound sutured and topical local  
309 anaesthetic (lidocaine/prilocaine) was applied. Mice were recovered on a heated blanket for 4-6h  
310 and then returned to normal housing. After a 3 day recovery, mice were anaesthetised and perfused  
311 transcardially with saline followed by 4% paraformaldehyde. Brains were removed and rostral and  
312 caudal blocks prepared using a brain matrix (Harvard Apparatus). Blocks were post-fixed in 4%  
313 paraformaldehyde for 24 h and processed to paraffin blocks. Sections (6µm) were cut on a  
314 microtome (Leica) and stained with haematoxylin and eosin. Ischaemic neuronal death was  
315 quantified in the CA1 and CA2 regions of the hippocampus which are the most sensitive regions in  
316 this model. Ischaemic (dead) neurons were identified morphologically in two regions of interest  
317 (ROIs) in CA1 and the entire CA2 bilaterally. Data are expressed as the number of dead neurons as

a % of total neurons in the ROI and show the mean of both hemispheres for each region. All processing and analysis was performed with the operator blind to genotype.

### **Statistical analysis, equipment and settings.**

Statistical testing involved a 2-tailed paired Student's t-test, or a one- or two-way ANOVA followed by an appropriate post-hoc test, as indicated in the legends. Cell death analyses for both *in vitro* and *in vivo* experiments were performed blind to the genotype/experimental condition. For all cell death, western blot analyses and *in vitro* and *in vivo* cell death experiments, the value of 'N' was taken as the number of independent biological replicates, defined as independently performed experiments on material derived from different animals. For western blots, we used chemiluminescent detection on Kodak X-Omat film, and linear adjustment of brightness/contrast applied (Photoshop) equally across the image, maintaining some background intensity. In any cases where lanes from non-adjacent lanes are spliced together, lanes are always from the same blot, processed in the same way, and the splicing point is clearly marked. Pictures of cells were taken on a Leica AF6000 LX imaging system, with a DFC350 FX digital camera.

### **Acknowledgements**

We thank Michelle Stewart, Roland Quinney and the team at MRC Harwell, the wider International Mouse Phenotyping Consortium, and the MRC Neurodegenerative Processes of Ageing and Disease (nPAD) mouse network for the generation, supply and import of *Dapk<sup>-/-</sup>* mice. We also thank Kathryn Elsegood and David Fricker for mouse colony management and genotyping. This work is funded by the MRC, Alzheimer's Research UK, Alzheimer's Society, the BBSRC (Roslin Institute strategic programme grant - BB/J004332/1), the Wellcome Trust and the European Commission.

### **Ethics**

Animal experimentation: Animals used in this study were treated in accordance with UK Animal Scientific Procedures Act (1986) and the work subject to local ethical review approval by the University of Edinburgh Ethical Review Committee. The relevant Home Office project licences are P1351480E and 60/4407, and the use of genetically modified organisms approved by local committee reference SBMS 13\_007.

### **Competing Financial Interests**

The authors declare no competing financial interests in this study.

### **Supplementary Material:**

*Figure Supplements:*

356 Figure 1–figure supplement 1a-h  
 357 Figure 3–figure supplement 1a-c  
 358  
 359 *Source Data:*  
 360 Figure 1–source data 1. Data relating to Figure 1b  
 361 Figure 1–source data 2. Data relating to Figure 1d  
 362 Figure 1–source data 3. Data relating to Figure 1f  
 363 Figure 1–source data 4. Data relating to Figure 1–figure supplement 1a  
 364 Figure 1–source data 5. Data relating to Figure 1–figure supplement 1f  
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 366 Figure 2–source data 1. Data relating to Figure 2a  
 367 Figure 2–source data 2. Data relating to Figure 2b  
 368 Figure 2–source data 3. Data relating to Figure 2c  
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 370 Figure 2–source data 5. Data relating to Figure 2e-g  
 371 Figure 3–source data 1. Data relating to Figure 3a  
 372 Figure 3–source data 2. Data relating to Figure 3e  
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 374 Figure 3–source data 4. Data relating to Figure 3–figure supplement 1a  
 375 Figure 3–source data 5. Data relating to Figure 3–figure supplement 1b

376

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## Figure Legends

**Figure 1. Neither Dapk1 nor excitotoxic insults increase GluN2B phosphorylation on Ser-1303. A,B)** Strong excitotoxic insults induce GluN2B Ser-1303 dephosphorylation at later timepoints. Western analysis of extracts from cortical neurons treated as indicated with NMDA or bicuculline (50  $\mu$ M) plus 4-amino pyridine (250  $\mu$ M). (F(2,24)=3.904, P=0.034 (Two-way ANOVA). \*P= 0.0053 (Sidak's post-hoc test; 95% CI of diff: 0.1777 to 1.139, comparison to control without NMDA treatment, N=3). **C,D)** Mimicking ischemic conditions triggers dephosphorylation of GluN2B Ser-1303 in an NMDAR-dependent manner. Oxygen-glucose deprivation (OGD) applied for 120 min  $\pm$  MK-801 (10  $\mu$ M). F(1,12)=6.69, P=0.024 (Two-way ANOVA). \*P= 0.0003 (Sidak's post-hoc test, 95% CI of diff: 0.3289 to 0.9172, N=4). **E,F)** Dapk1 deficiency does not influence basal or NMDA-induced GluN2B Ser-1303 phosphorylation status. Neurons were treated  $\pm$  50  $\mu$ M NMDA for 60 min. F(1,10)=345.1, P<0.0001 (Two-way ANOVA, Con vs. NM). \*P<0.0001 (both, compared to Con of that genotype, 95% CI of diff: 0.6384 to 0.9342, and 0.6411 to 0.9826 (reading left to right), N=4 WT, N=3 KO; with "N" defined as a distinct culture from a distinct animal). ns: F(1,10)=0.5418, P=0.4786.

**Figure 2. Excitotoxic and ischemic insults are not ameliorated by Dapk1 deficiency. A, B)** NMDA-induced neuronal death is independent of Dapk1. Cortical neurons at DIV10 (A) or DIV16 (B) were treated as indicated for 1 h, with neuronal death assessed at 24 h. The p values relate to a two-way ANOVA test of differences between WT and Dapk<sup>-/-</sup> neurons (F(1,10) = 0.2676, n=6 WT, 6 KO (DIV10); F(1,7)=0.8871, 2-way ANOVA, n=4 WT, 5 KO (DIV16)). For each condition/genotype combination, 800-1000 cells were analysed per biological replicate. **C)** OGD-induced neuronal death is independent of Dapk1. Cortical neurons at DIV10 were subjected to OGD for 120 min, before being returned to control medium. Neuronal death was assessed at 24 h. No genotype-dependent difference was observed (F (1,12)=0.5062, P=0.490, but a strong influence of OGD was observed: F (1,12) = 63.54, P<0.0001, two-way ANOVA. #P=0.0002, 0.0002 (reading left to right); Sidak's post-hoc test comparing control to OGD condition (n=4 WT, n=4 KO). **D)** Dapk1 deficiency does not influence NMDAR currents. NMDAR currents were measured in n=16 WT cells (from 4 separate cultures) and n=25 KO cells (from 6 separate cultures). Currents were normalized to the mean current recorded from WT cells recorded on that precise day. P=0.411 (t=0.831, df=39), unpaired t-test. **E-G)** Dapk1 deficiency does not influence vulnerability to ischemia *in vivo*. Adult age-matched mice (n=14 WT; n=16 KO) were subjected to 20 min bilateral common carotid artery occlusion, sacrificed at 3 d, and pathology analysed. CA1/2 (E): P=0.555 (t=0.598, df=28); CA1 (F):P=0.572 (t=0.572, df=28), CA2 P=0.592(G, t=0.543, df=28). Scale bar = 50  $\mu$ m.

537

538 **Figure 3. Both TAT-NR2B<sub>CT</sub> and TAT-sNR2B<sub>sCT</sub> are direct NMDAR antagonists. A-D)** Both  
 539 TAT-NR2B<sub>CT</sub> and TAT-sNR2B<sub>CT</sub> (scrambled version of TAT-NR2B<sub>CT</sub>) immediately antagonize  
 540 NMDAR currents upon extracellular exposure. NMDA-induced currents were recorded under whole-  
 541 cell voltage clamp, with the indicated peptides (at 5  $\mu$ M) applied approximately 5 s after NMDA (to  
 542 allow NMDAR currents to reach steady state). Arg-rich refers to the arginine-rich positively charged  
 543 peptide; Neutral refers to the neutral peptide-see main text for sequences of these as well as TAT-  
 544 NR2B<sub>CT</sub> and TAT-sNR2B<sub>CT</sub>. NMDA-induced NMDAR currents were monitored for a further 45 s and  
 545 the percentage drop in currents calculated, compared to no peptide at all (Con) which represents a  
 546 measure of natural desensitization over this period.  $P < 0.0001$  (one-way ANOVA). \* $P < 0.0001$ ,  
 547 Sidak's post-hoc test ( $n = 8$  of all conditions). Example traces shown in (B) (C) and (D). Scale bar: 15  
 548 s, 500 pA. **E)** NMDAR antagonism by TAT-NR2B<sub>CT</sub> is inhibited by  $Mg^{2+}$  blockade. NMDAR currents  
 549 were measured, after which neurons were incubated in TAT-NR2B<sub>CT</sub> (5 or 0.5  $\mu$ M) for 60 s in the  
 550 presence or absence of 1 mM  $Mg^{2+}$ , after which NMDAR currents were measured again (in zero  
 551  $Mg^{2+}$ , no peptide).  $P < 0.0001$  (one-way ANOVA,  $F(1,22) = 47.16$  (effect of  $[Mg^{2+}]$ )). \* $P < 0.0001$ ,  
 552  $P = 0.0007$ , Sidak's post-hoc test (zero  $Mg^{2+}$ :  $n = 6$  (0.5  $\mu$ M),  $n = 7$  (5  $\mu$ M); 1 mM  $Mg^{2+}$ :  $n = 6$  (0.5  $\mu$ M),  
 553  $n = 7$  (5  $\mu$ M)). **F-H)** NMDAR antagonism by TAT-NR2B<sub>CT</sub> is more effective on open channels. See  
 554 main text for experimental details.  $P < 0.0001$  (two-way ANOVA, comparing initial current with  
 555 subsequent measurements:  $F(2, 45) = 25.22$ .  $P < 0.0001$  (two-way ANOVA, comparing 'closed-then-  
 556 closed' protocol (grey bars,  $n = 8$ ) with 'closed-then-open' protocol (black bars,  $n = 9$ ):  $F(1, 45) =$   
 557  $18.26$ . # $P = 0.003$ , 0.0009 (Sidak's post-hoc test), comparing to initial currents. \* $P < 0.0001$  (Sidak's  
 558 post-hoc tests), comparisons indicated. **(G)** shows example recordings taken during the consecutive  
 559 "closed" then "closed" channel protocol. **(H)** shows example recordings taken during the  
 560 consecutive "closed" then "closed" channel protocol. Scale bar = 1 s, 250 pA.

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### Figure Supplement Legends

565

566 **Figure 1—figure supplement 1. A,B)** Neurons were treated with staurosporine (STS, 1  $\mu$ M) or FK-  
 567 506 (FK, 5  $\mu$ M) + okadaic acid (OA, 10  $\mu$ M) for one hour, after which protein was harvested and  
 568 western analysis for Phospho- (GluN2B Ser-1303) levels performed.  $P = 0.0023$  (1-way ANOVA).  
 569 Individual P-values left-to-right: 0.026, 0.047 ( $n = 8$  (FK+OA);  $n = 4$  (STS). **C)** Schematic depicting the  
 570 amino-acid changes resulting from mutations of the *Grin2b* gene in the GluN2B <sup>$\Delta$ CaMKII</sup> allele. **D)**  
 571 Example Phospho- (GluN2B Ser-1303) western blot illustrating the lack of immunoreactivity of the  
 572 mutated domain in extracts from GluN2B <sup>$\Delta$ CaMKII/ $\Delta$ CaMKII</sup> neurons. **E,F)** timecourse of GluN2B Ser-1303  
 573 phosphorylation status in response to NMDA treatment (50  $\mu$ M).  $F(5,10) = 4.019$ ,  $P = 0.023$  (one-way  
 574 ANOVA). \* $P = 0.041$  (Sidak's post-hoc test ( $n = 3$ ), 95% CI of diff 0.01897 to 1.024). **G,H)** Experiment



575 performed as per Figure 1a except neurons were at DIV16 rather than DIV10.  $F(2, 24) = 5.324$ ,  
576  $P=0.0122$  (two-way ANOVA). \* $P=0.0022$  (Sidak's post-hoc test ( $N=3$ )).

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578

579 **Figure 3—figure supplement 1. A)** Neurons were treated where indicated with 50  $\mu\text{M}$  TAT-NR2B<sub>CT</sub>  
580 for 1 h, with death assessed after 24 h. **B)** Neurons were pre-treated where indicated with TAT-  
581 NR2B<sub>CT</sub> or TAT-sNR2B<sub>CT</sub> for 1 h, prior to 1h NMDA treatment at the indicated concentrations, in the  
582 continued presence of the peptides where used. Subsequently, both NMDA and peptide were  
583 removed from the medium and death assessed after a further 23 h.  $P<0.0001$  (effect of peptide:  
584 two-way ANOVA,  $F(2,40)=76.13$ ,  $n=3-4$ ). \* $P= 0.0027$ ,  $0.0039$  (20  $\mu\text{M}$ ),  $<0.0001$ ,  $<0.0001$  (30  $\mu\text{M}$ ),  
585  $<0.0001$ ,  $<0.0001$  (50  $\mu\text{M}$ ),  $<0.0001$ ,  $<0.0001$  (100  $\mu\text{M}$ ), Sidak's post-hoc test. **C)** Example trace  
586 from an experiment where NMDA-induced currents were recorded under whole-cell voltage clamp,  
587 with NR2B<sub>CT</sub>(1292-1304)-TAT (5  $\mu\text{M}$ , Merck Millipore) applied approximately 5 s after NMDA (to  
588 allow NMDAR currents to reach steady state). NMDA-induced NMDAR currents were monitored to  
589 determine the degree of blockade. The trace is representative of 8 cells recorded this way, with the  
590 peptide blocking by  $63 \pm 3 \%$ . Scale bar = 5 s, 500 pA.

591

592

#### Source Data Legends

593 **Figure 1—source data 1.** Data relating to Figure 1b

594 **Figure 1—source data 2.** Data relating to Figure 1d

595 **Figure 1—source data 3.** Data relating to Figure 1f

596 **Figure 1—source data 4.** Data relating to Figure 1—figure supplement 1a

597 **Figure 1—source data 5.** Data relating to Figure 1—figure supplement 1f

598 **Figure 1—source data 6.** Data relating to Figure 1—figure supplement 1h

599 **Figure 2—source data 1.** Data relating to Figure 2a

600 **Figure 2—source data 2.** Data relating to Figure 2b

601 **Figure 2—source data 3.** Data relating to Figure 2c

602 **Figure 2—source data 4.** Data relating to Figure 2d

603 **Figure 2—source data 5.** Data relating to Figure 2e-g

604 **Figure 3—source data 1.** Data relating to Figure 3a

605 **Figure 3—source data 2.** Data relating to Figure 3e

606 **Figure 3—source data 3.** Data relating to Figure 3f

607 **Figure 3—source data 4.** Data relating to Figure 3—figure supplement 1a

608 **Figure 3—source data 5.** Data relating to Figure 3—figure supplement 1b

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Figure 1

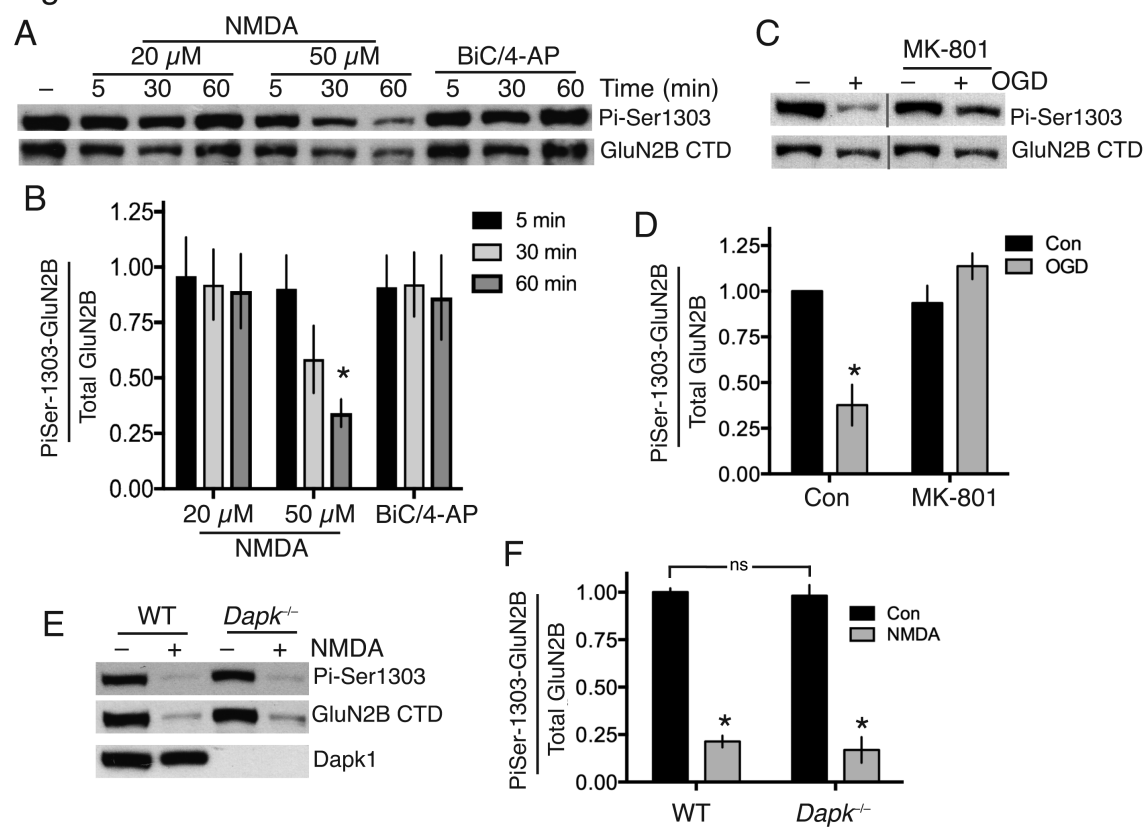


Figure 2

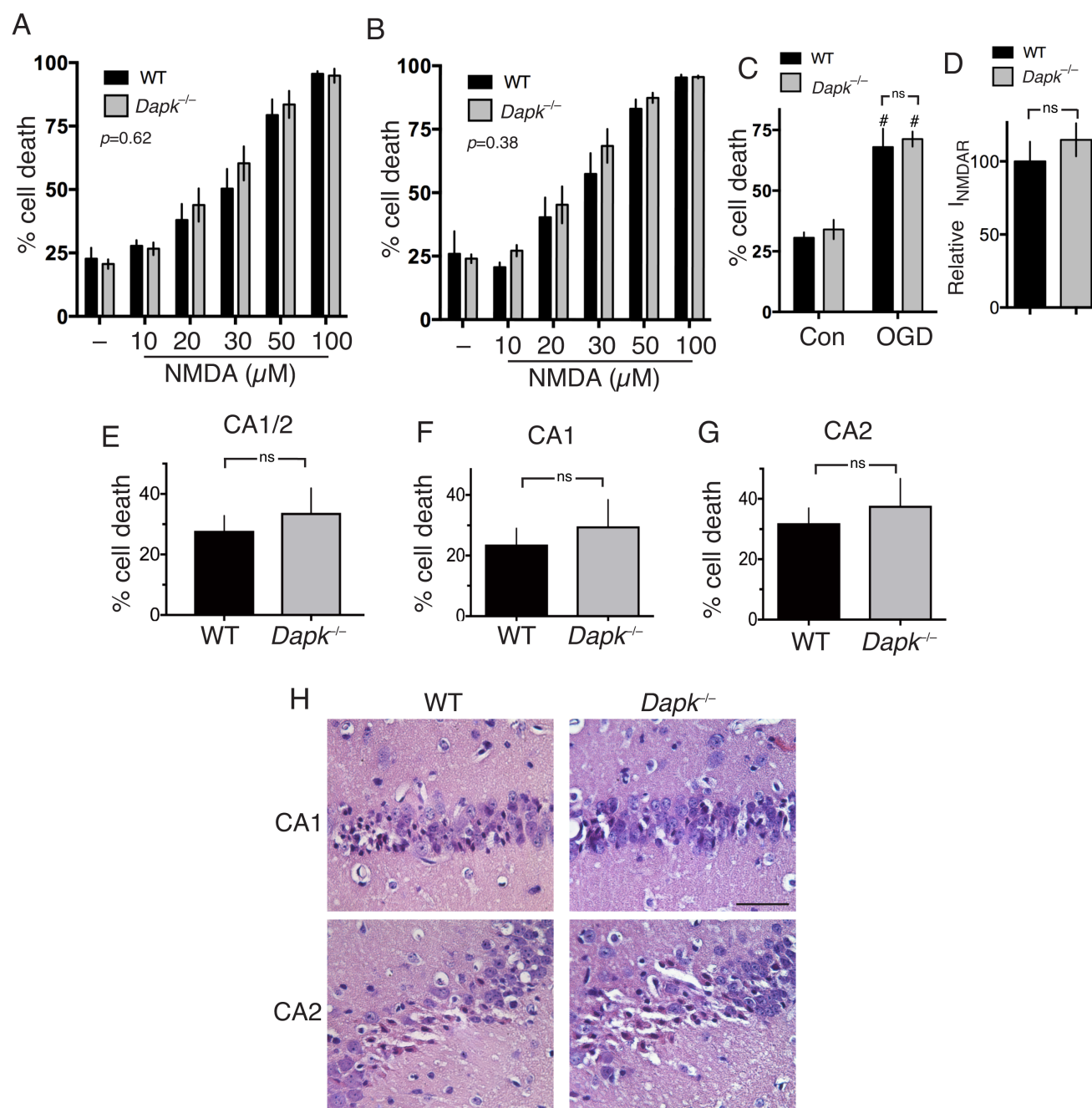


Figure 3

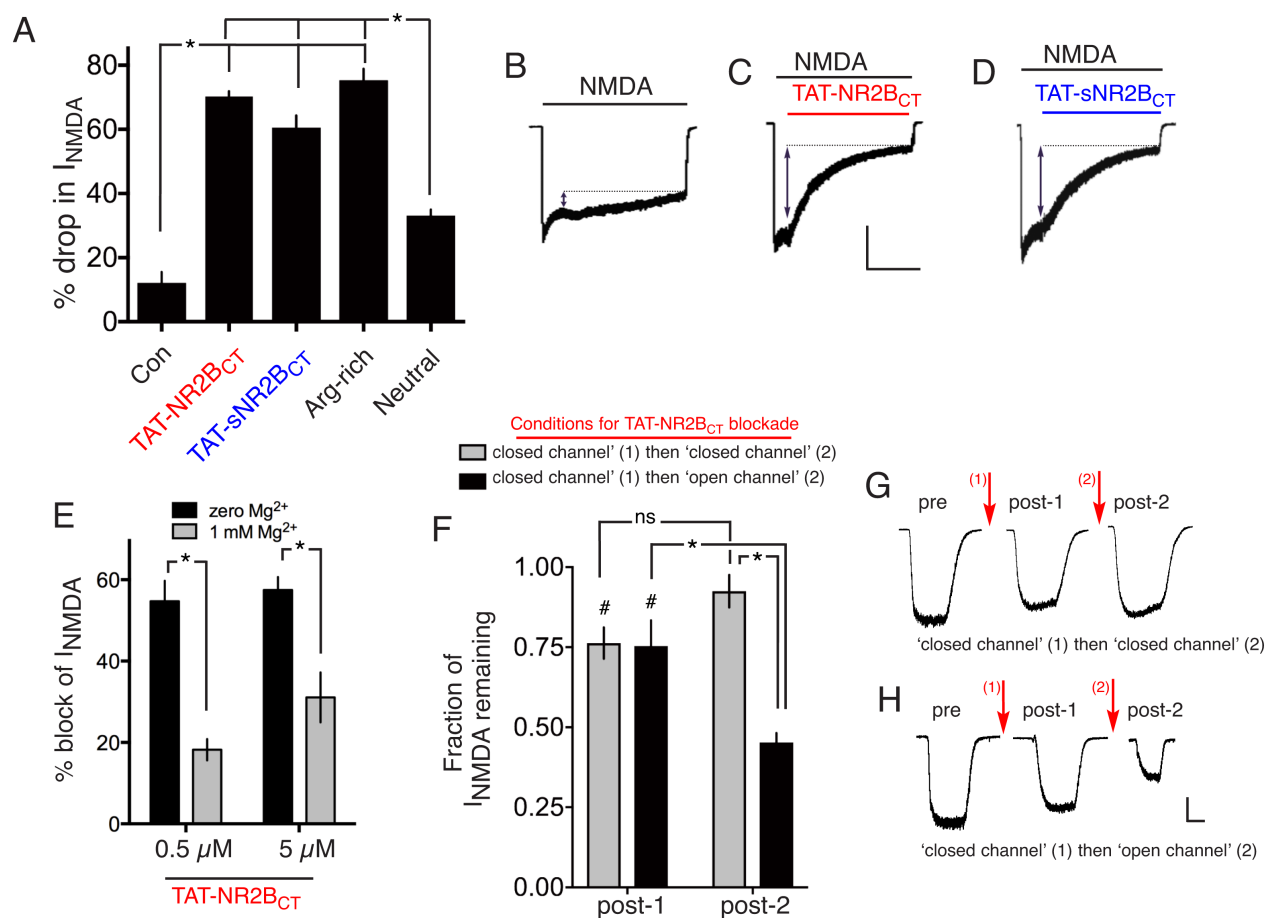


Figure 1–figure supplement 1

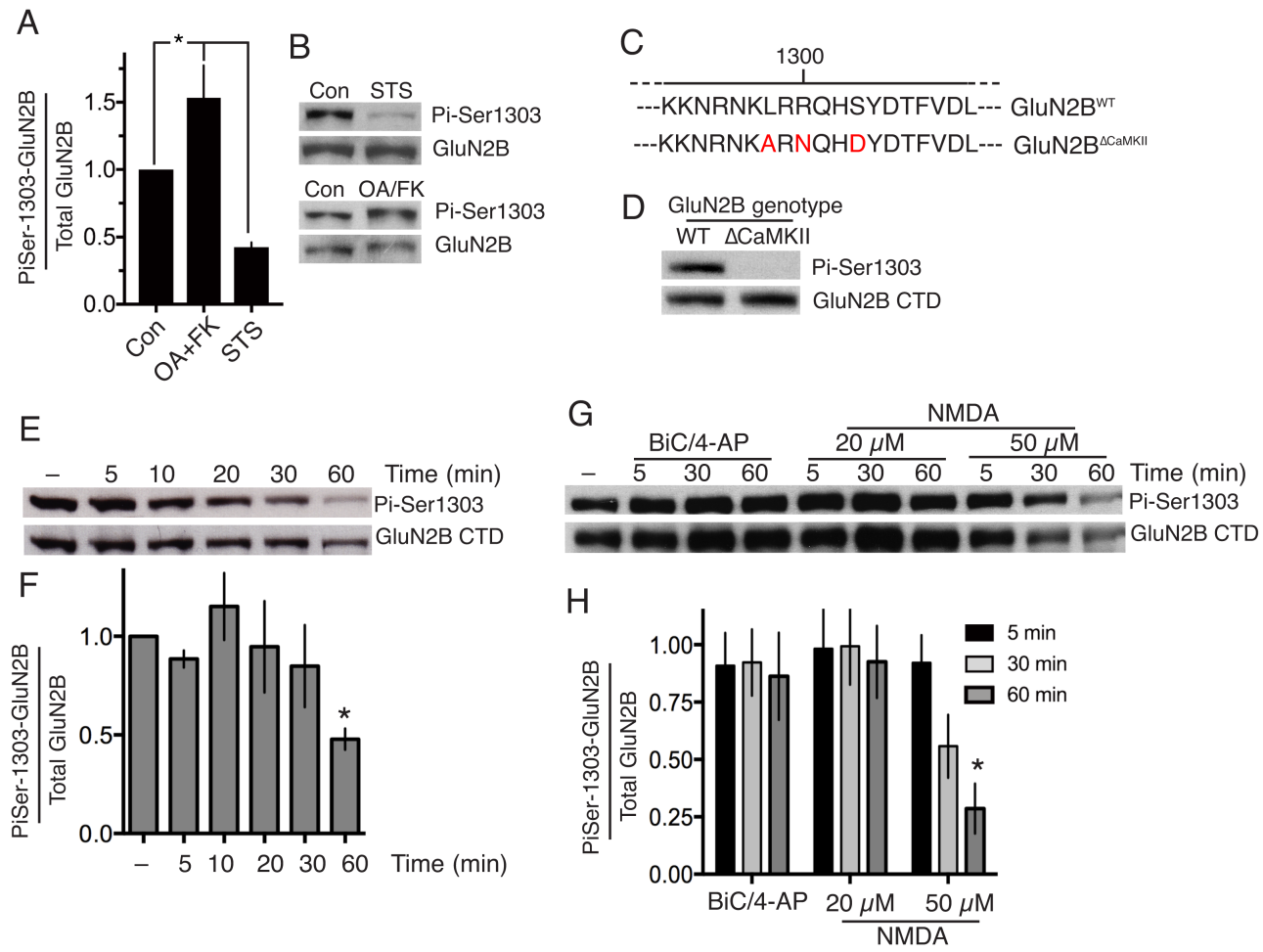


Figure 3—figure supplement 1

